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Regulation of glutamate level in rat brain through activation of glutamate dehydrogenase by *Corydalis ternata*

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Abbreviations: *C. ternata, Corydalis ternata*; GDH, glutamate dehydrogenase

Abstract

When treated with protopine and alkalized extracts of the tuber of *Corydalis ternata* for one year, significant decrease in glutamate level and increase in glutamate dehydrogenase (GDH) activity was observed in rat brains. The expression of GDH between the two groups remained unchanged as determined by Western and Northern blot analysis, suggesting a post-translational regulation of GDH activity in alkalized extracts treated rat brains. The stimulatory effects of alkalized extracts and protopine on the GDH activity was further examined *in vitro* with two types of human GDH isozymes, hGDH1 (house-keeping GDH) and hGDH2 (nerve-specific GDH). Alkalized extracts and protopine activated the human GDH isozymes up to 4.8-fold. hGDH2 (nerve-specific GDH) was more sensitively affected by 1 mM ADP than hGDH1 (house-keeping GDH) on the activation by alkalized extracts. Studies with cassette mutagenesis at ADP-binding site showed that hGDH2 was more sensitively regulated by ADP than hGDH1 on the activation by *Corydalis ternata*. Our results suggest that prolonged exposure to *Corydalis ternata* may be one of the ways to regulate glutamate concentration in brain through the activation of GDH.

Keywords: glutamate dehydrogenase; glutamates; plant extracts; plants, medicinal; protopine

Introduction

Glutamate dehydrogenase (GDH) (EC 1.4.1.3) is a family of enzymes catalyzing a reversible deamination of L-glutamate to α-ketoglutarate. The importance of the pathophysiological nature of the GDH-deficient neurological disorders has attracted considerable interest (Plaitakis *et al*., 1982). The enzyme isolated from one of the patients with a variant form of multisystem atrophy displayed a marked reduction of one of the GDH isozymes (Hussain *et al*., 1989). It also has been reported that inhibition of GDH expression by antisense oligonucleotides was toxic to cultured mesencephalic neurons with dopaminergic neurons being affected at the early stages of this inhibition, suggesting an important role of GDH in central nervous system (Plaitakis and Shashidharan, 2000). It was reported that the presence of four differently sized mRNAs and multiple gene copies for GDH in the human brain (Mavrothalassitis *et al*., 1988). Previous studies have shown that hGDH1 (house-keeping GDH) encoded by the gene *GLUD1* is expressed widely, whereas hGDH2 (nerve-specific GDH) encoded by the gene *GLUD2* is specific for neural and testicular tissues (Shashidharan *et al*., 1994). Most recent studies have reported that *GLUD2* originated by retroposotion from *GLUD1* in the hominoid ancestor less than 23 million yrs ago and the amino acid changes responsible for the unique nerve-specific properties of the enzyme derived from *GLUD2* occurred during a period of positive selection after the duplication event (Burki and Kaessmann, 2004). However, the functional significance of nerve-specific GDH isotype in nerve tissue remains to be studied.

We have been searching for GDH regulators from natural resources such as medicinal plants by screening extracts for GDH activity. We found that methanolic extracts of the tuber of *Corydalis ternata* Nakai (Papaveraceae) showed stimulatory effects on GDH activity. The *C. tubers* have been traditionally used as lenitive and used to treat cardiovascular diseases such as hypertension and cardiac arrhythmia in oriental countries. Several studies have

shown that active components in *Corydalis* have anti-cholinesterase and anti-amnesic activities, anti-inflammatory activities, anti-hypertensive effects, and analgesic effects (Kubo *et al*., 1994; Kim *et al*., 1999; Chang and Lin, 2001). Other studies also have shown that *C. tubers* deplete the levels of amygdaloid dopamine (Liu *et al*., 1982) and have neuroprotective effects in heat-stroke rats (Chang *et al*., 1999). Previously, Ito *et al*. (2001) have suggested that the isoquinoline alkaloids isolated from *Corydalis* species may act as anti-tumor promoters based on their observations that the alkaloids show inhibitory effects on Epstein-Barr virus early antigen activation induced by 12-*O*-tetradecanoylphorbol 13-acetate in Raji cells.

One of the well-documented components of the extracts of *Corydalis ternata* is protopine. Protopine has been known to regulate activity on elastase (Tanaka *et al*., 1993) and aggregation of platelets (Matsuda *et al*., 1988) and to affect cholinergic systems on isolated guinea-pig ileum (Ustunes *et al*., 1988). It has been reported that protopine inhibits the high-potassium induced, Ca^{2+}-dependent contraction of rat aorta (Ko *et al*., 1992) and increases GABA binding to rat brain synaptic membranes (Kardos *et al*., 1986). The potential of protopine to inhibit microsomal drug metabolizing enzymes and to prevent paracetamol- and CCl_4-induced hepatotoxicity in rats was reported (Janbaz *et al*., 1998). In addition, protopine has been shown to have multiple actions on cardiovascular system, including anti-arrhythmic, anti-hypertensive and negative inotropic effects (Song *et al*., 2000).

In the present work, we present the effects of protopine and alkalized extracts of the *C. ternata* on GDH *in vivo* and *in vitro* in view of the central role of GDH in cerebral metabolism.

Materials and Methods

Materials

NADH, NAD^+, α-ketoglutarate, glutamate, ADP, and protopine hydrochloride were purchased from Sigma Chemical Co. Stock solution (10 mM) of protopine was prepared in distilled water, kept at 4°C. Human GDH genes (pHGDH1 and pHGDH2) have been chemically synthesized, expressed in *E. coli* as soluble proteins, and homogeneously purified by the same methods developed in our laboratory as described elsewhere (Cho *et al*., 2001; Lee *et al*., 2001; Yoon *et al*., 2002a; Yang *et al*., 2004). Y187M mutant at the ADP-binding site of the hGDH isozymes was constructed and homogeneously purified by the same methods developed in our laboratory as described elsewhere (Cho *et al*., 2001; Yoon *et al*., 2002b). All other chemicals and solvents were reagent grade or better.

Isolation of alkalized extracts from *C. ternata*

C. ternata was purchased from Kyungdong Market, Seoul, Korea and identified in our laboratory. Dried *C. ternata* was extracted 6 times with methanol in an ultrasonic that yielded a methanolic extract. The methanolic extract was suspended in H_2O and partitioned successively with CH_2Cl_2 followed by 0.15% HCl solution as described elsewhere (Kim *et al*., 1999). The HCl fraction was alkalized with 10% Na_2CO_3, repartitioned with CH_2Cl_2, and used as the alkalized extracts of *C. ternata* for this study.

Enzyme assay and activation studies

GDH activity was measured spectrophotometrically as described before (Choi *et al*., 1999; Jang *et al*., 2003) except that no ADP was added unless otherwise indicated. One unit of enzyme was defined as the amount of enzyme required to oxidize 1 mM of NADH per min at 25°C. Stimulation studies with alkalized extracts or protopine were performed at various concentrations in assay buffer at 25°C as described in figure legends. In some experiments, the effects of ADP on activation of hGDH isozymes by alkalized extracts were examined as described in the figure legends.

Effects of alkalized extracts on rat brains

Male Sprague-Dawley rats (10 weeks old) were divided into control group, protopine-treated group (10 μg/g body weight) and alkalized extracts-treated group (100 mg/g body weight), and fed ad libitum standard laboratory diet. All animal procedures were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals. After 1 yr, the animals were killed by decapitation and brains were dissected, rinsed with 0.9% ice-cold saline, weighed, and homogenized in 50 mM Tris/HCl, pH 7.4 containing 0.5 mM ethylenediaminetetraacetate and 1.0 mM β-mercaptoethanol. The homogenates were centrifuged at 8,000 *g* for 40 min at 4°C and the supernatants were assayed for protein, total DNA and RNA, and the activities of GDH. Ammonia, ornithine, α-ketoglutarate, glutamate and other amino acids concentrations were measured in neutralized brain extracts by enzymatic and fluorometric methods (Heinrikson and Meredith, 1984).

Northern and Western blots

The 8,000 *g* supernatants were applied to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane for Western blot analysis (Kim *et al*., 2004). The monoclonal

antibodies used in this study were produced against bovine brain GDH as described before in our laboratory (Choi *et al*., 1999; Jang *et al*., 2003). For the isolation of RNA, five volumes of 4M guanidinium thiocyanate buffer were added to brains and homogenized with a Polytron homogenizer. Hybridization was performed by adding saturating amounts of ^{32}P-labeled and denatured cDNA probes along with 100 μg/ml denatured salmon sperm DNA. Autoradiography was performed overnight at -80°C. Intensity of the bands was densitometrically determined and normalized to the intensity of the corresponding α-tubulin bands.

Statistical analysis

The evaluation of statistical significance was determined by an ANOVA test. The data were considered to be statistically significant if the probability had a value of 0.05 or less.

Results and Discussion

To examine the long-term effects of *C. ternata* on glutamate level and activities of the enzymes involved in glutamate metabolism in rat brain, rats were kept in equal groups with or without protopine and alkalized extracts for one yr. There were no significant changes in the body weight, brain weight, and protein contents after one yr of feeding (Table 1). Also, the RNA and DNA content did not vary in the three groups. The alkalized extracts treated group showed a brain glutamate concentration of 18 μM/g wet weight that were 48% lower than the control group (Table 1). In contrast, the concentrations of NH_4^+ and α-ketoglutarate in the alkalized extracts treated group were 39% and 41% higher than those in the control group, respectively (Table 1). Although the effects of protopine was less than those of alkalized extracts, protopine also reduced glutamate up to 23% and increased the concentrations of NH_4^+ and α-ketoglutarate approximately 33% and 31%, respectively (Table 1). There were no differences between the groups in the concentrations of other amino acids.

The effects of *C. ternata* on activities of the enzymes involved in glutamate metabolism in rat brain were examined. A significant increase in the GDH activity was observed after one year of feeding the alkalized extracts (Table 1). The specific activities were increased 2.4-fold in the alkalized extracts treated group (0.84 U/mg protein) and 1.6-fold in the protopine treated group (0.57 U/mg protein) compared to those in the control group (0.35 U/mg protein), whereas no significant changes were observed in K_m values between the groups (Table 1). The results in Figure 1 show that there were no significant differences in the expression level of GDH among the groups as determined by the Western blot analysis and Northern blot analysis. These results suggest a post-translational regulation of GDH activity in the alkalized extracts and protopine treated rat brains. Since protopine showed relatively lower effects than alkalized extracts on glutamate level and GDH activity, it seems likely that other factors of the extracts, in addition to protopine, may be necessary to show the maximal effects of *C. ternata*. Alkalized extracts and protopine up to 200 μg/ml and 500 μM,

Table 1. Effects of protopine and alkalized extracts feeding on the glutamate content and the activity of GDH in rat brains.

Parameters	Control group (n = 4)	Protopine treated group (n = 4)	Alkalized extracts treated group (n = 4)
Body weight (g)	635 ± 31	631 ± 29	647 ± 43
Brain weight (g)	2.3 ± 0.3	2.2 ± 0.3	2.3 ± 0.2
Protein (mg/g wet tissue)	224 ± 11	218 ± 13	229 ± 16
Glutamate (μM/g wet weight)	35.11 ± 0.72	27.05 ± 1.10*	18.36 ± 0.83*
α-Ketoglutarate (μM/g wet weight)	9.67 ± 0.61	12.67 ± 0.62*	13.65 ± 0.77*
NH_4^+ (μM/g wet weight)	1.36 ± 0.19	1.81 ± 0.11*	1.89 ± 0.15*
Specific activity (U/mg protein)	0.35 ± 0.02	0.57 ± 0.04*	0.84 ± 0.03*
$K_{m(NADH)}$ (μM)	36.09 ± 1.04	37.85 ± 0.89	38.33 ± 0.96
$K_{m(Ammonia)}$ (mM)	6.13 ± 0.79	5.40 ± 0.83	5.28 ± 0.88
$K_{m(Glutamate)}$ (mM)	3.21 ± 0.24	2.99 ± 0.28	3.02 ± 0.15
$K_{m(\alpha\text{-Ketoglutarate})}$ (mM)	1.38 ± 0.13	1.31 ± 0.19	1.11 ± 0.24

Values are expressed as means ± S.D. for four rats with all measurements performed in duplicate.
*Significantly different from the control group, $P < 0.05$.

respectively, did not show any effects on the activities of glutamine synthetase, glutamate decarboxylase, and α-ketoglutarate dehydrogenase, which are involved in glutamate metabolism (data not shown). These results may suggest an expectation to have no significant side effects attributable to non-specific cross-reactivity to other mitochondrial enzymes.

Unlike to synaptic glutamate transporters, most glutamate-degrading enzymes have much lower affinities for glutamate, but higher capacity for glutamate elimination. The known glutamate-degrading enzymes such as glutamine synthetase, GDH, and glutamate pyruvate transaminase have already been shown to have neuroprotective value in models of glutamate excitotoxicity (Matthews *et al*., 2000). GDH has been suggested to be associated with the pool of glutamate that is released as a neurotransmitter at the nerve endings (Aoki *et al*., 1987). The precise roles of GDH in the central nervous system as well as the predominant direction of the reaction it catalyzes remain unclear. However, given the extensive nature of the glutamatergic pathways in brain (Aoki *et al*., 1987), GDH may play a role in a no., of human neurodegenerations. Subsequently decreased catabolism of glutamate at the nerve terminals could result in an increased amount of the neurotransmitter at the synapses, leading to over-excitation and neuronal degeneration. The studies on specific glutamate antagonists also suggested the possibility that glutamate excitotoxicity might be involved in epilepsy (Mathern *et al*., 1999). Therefore, the regulation of GDH that is important in adjusting the levels of the neurotransmitter glutamate might be worth examining in the context of experimental and clinical neurodisorders.

Figure 1. Effects of alkalized extracts feeding on the expression of GDH in rat brains. (A) Western blot. The supernatants of the crude extracts were immunoblotted with the monoclonal antibody directed against bovine brain GDH [20]. (B) Northern blot analysis. GDH mRNA levels were compared and normalized against α-tubulin. Lanes 1-4, control group; lanes 5-8, protopine treated group; lanes 9-12, alkalized extracts treated group. Densitometric analyses showed no significant differences in protein expression and mRNA level among the groups.

The effects of alkalized extracts and protopine on the GDH activity were further examined with two different types of human GDH isozymes, hGDH1 (house-keeping GDH) and hGDH2 (nerve-specific GDH). When the effects of varied alkalized extracts at various concentrations on the activities of hGDH isozymes were studied, a marked activation was observed up to 3.2-fold and 4.1-fold for hGDH1 and hGDH2, respectively (Figure 2A). hGDH2 was more activated than hGDH1 and the half-maximal stimulation was reached at 35 μg/ml and 22 μg/ml for hGDH1 and hGDH2, respectively (Figure 2A). Protopine also activated both hGDH isozymes but to a less extent (Figure 2B).

Most distinctive differences in their sensitivity to stimulation by alkalized extracts between hGDH1 and hGDH2 were observed in the presence of ADP. It has been well-known that the activity of GDH is strictly controlled by allosteric regulators such as ADP

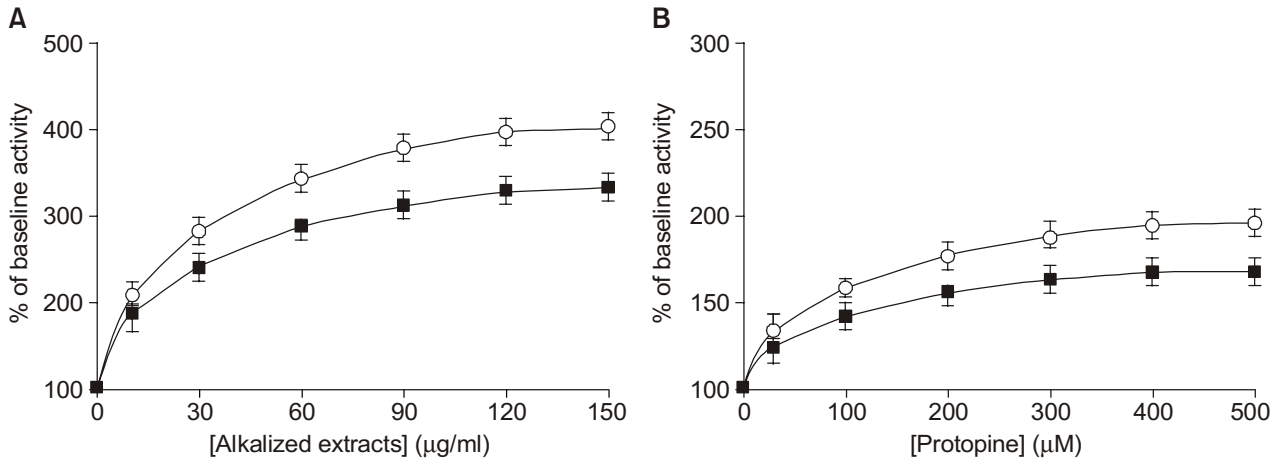

Figure 2. Effects of alkalized extracts and protopine on the activities of hGDH isozymes. hGDH isozymes were assayed and relative activities are expressed as percentage of baseline activity. (A) Alkalized extracts; (B) Protopine. ■ (hGDH1); ○ (hGDH2).

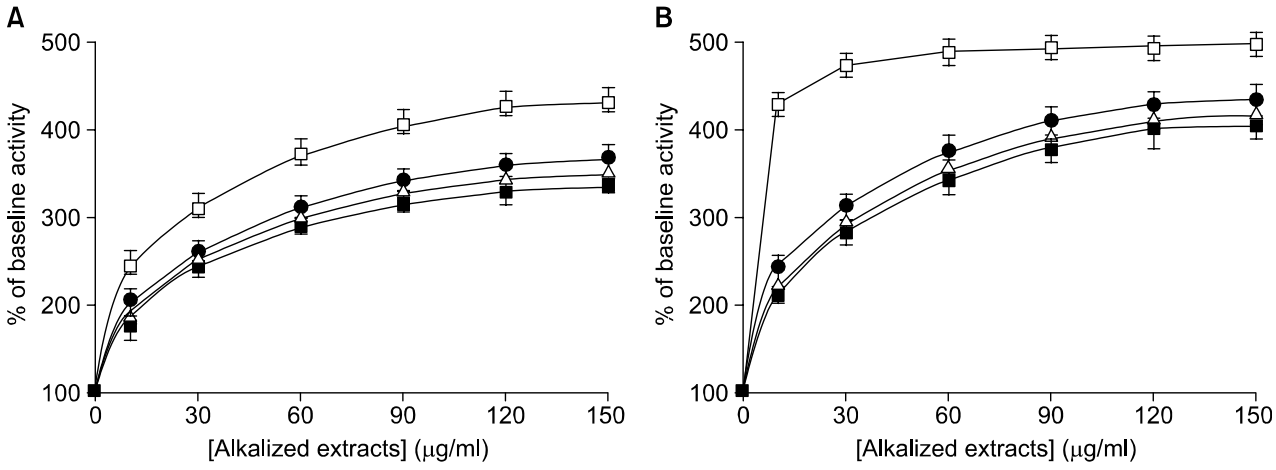

Figure 3. Effect of alkalized extracts on sensitivities of wild-type and Y187M mutant enzymes to ADP. Wild-type and Y187M mutant enzymes were in the absence and presence of ADP. The relative activities are expressed as percentage of baseline activity. (A) hGDH1; (B) hGDH2. ■ (wild-type without ADP); ● (wild-type with 0.1 mM ADP); □ (wild-type with 1.0 mM ADP); Δ (Y187M mutant with or without 1.0 mM ADP).

(Bailey *et al*., 1982; Shashidharan *et al*., 1994). In the present study, we examined the role of ADP in sensitivities of hGDH isozymes to *C. ternata*. As shown in Figure 3, the activities of wild-type hGDH isozymes obtained at saturating concentration of alkalized extracts were increased further by subsequent addition of ADP. When low concentration of ADP (0.1 mM) was added, no appreciable differences in their stimulating activities by alkalized extracts were observed between hGDH1 (Figure 3A) and hGDH2 (Figure 3B). On the other hand, the sensitivities to alkalized extracts in the presence of high concentration of ADP (1.0 mM) were distinct for the two hGDH isozymes. hGDH2 (nerve-specific GDH) (Figure 3B) was more sensitively affected by 1 mM ADP than hGDH1 (house-keeping GDH) (Figure 3A) on the activation by alkalized extracts. In contrast to the wild-type hGDH isozymes, no further stimulation were observed for Y187M mutant by ADP and the results obtained with Y187M mutant without ADP were almost identical with those obtained with Y187M mutant in the presence of 1.0 mM ADP at all concentrations of alkalized extracts rested (Figure 3A and 3B). Similar effects of ADP on the different regulation of hGDH isozymes were also observed with protopine (data not shown). This result suggests that the regulation of GDH by ADP is mainly due to the binding of ADP to the Y187 residue for both hGDH isozymes as described elsewhere (Yoon *et al*., 2002b). It also indicates that the different sensitivities to alkalized extracts in the presence of high concentration of ADP may due to differences in the regulatory properties between hGDH isozymes by ADP. These observations are consistent with the previous reports that there are at least two different GDH activities differing in their relative thermal stability and allosteric regulation characteristics (Mavrothalassitis *et al*., 1988; Shashidharan *et al*., 1994; Cho *et al*., 2001; Yang et al., 2004). Since physiological ADP levels can vary from 0.05 to $>$1.0 mM depending on the rate of oxidative phosphorylation, our results suggest a possibility that hGDH1 and hGDH2 are differently regulated in vivo by the actions of alkalized extracts depending on the physiological concentrations of ADP.

Our results are consistent with the previous reports that there are at least two different GDH activities differing in their relative thermal stability and allosteric regulation characteristics (Bailey *et al*., 1982; Shashidharan *et al*., 1994). Since physiological ADP levels can vary from 0.05 to $>$1.0 mM depending on the rate of oxidative phosphorylation, our results suggest a possibility that hGDH1 and hGDH2 are differently regulated *in vivo* by the actions of *C. ternata* depending on the physiological concentrations of ADP. Activation of GDH provides an example where *C. ternata* biochemically mimics the action of ADP, although the potential physiological role of *C. ternata* remains to be answered. If ADP plays a significant role in a neurotransmitter function through different regulation of hGDH isozymes, the functional similarity between ADP and *C. ternata* may account for at least part of the pharmacological actions of *C. ternata*. Further studies of the similarities and differences between *C. ternata* and ADP in various CNS functions may provide more insight into the mechanism of action of *C. ternata*.

In conclusions, protopine and alkalized extracts of the tuber of *Corydalis ternata* decreased glutamate level and increased GDH activity in rat brains after

one yr treatment. Although further investigations are required to evaluate the precise molecular mechanism of the effects of *C. ternata* on glutamate contents in brain, our results suggest a possibility that the prolonged exposure to *C. ternata* may be one of the ways to regulate glutamate level in brain through the regulation of GDH, one of the central enzymes in glutamate metabolism.

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